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Visible Fluorescence Detection of Type III Protein Secretion from Bacterial Pathogens

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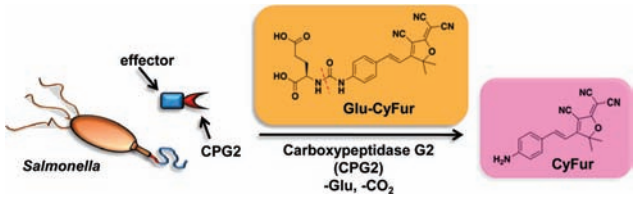
A number of Gram-negative bacterial pathogens utilize type III secretion systems (T3SSs) resembling molecular needle complexes to inject protein effectors into host cells[1] for invasion and intracellular replication.[2] T3SSs are essential for bacterial virulence, but the cellular factors that regulate T3SSs and protein secretion are not fully understood.[1] Recently described small molecules that block the secretion of bacterial effectors through T3SSs are promising leads for new antibacterials[3] but will require further development for clinical studies. Sensitive and high-throughput assays for type III protein secretion should facilitate mechanistic studies of T3SSs and discovery of small molecule inhibitors.[4] Herein, we describe a carboxypeptidase G2 (CPG2)-based reporter system for fluorescence and visible detection of type III protein secretion from Gram-negative bacterial pathogens such as *Salmonella enterica*[2] (Scheme 1).

Scheme 1. A Bacterial Secreted Effector-CPG2 Fusion Protein Reports on *Salmonella* Type III Secretion through Color and Fluorescence Change of Glu-CyFur



The orthogonal specificity of CPG2 presents an attractive enzyme reporter system to monitor bacterial protein secretion. CPG2 is a 43 kDa metalloprotease, found in rare *Pseudomonas syringae* strains, that selectively cleaves glutamate (Glu) from small molecule metabolites.[5] Importantly, the glutamyl-carboxypeptidase activity of CPG2 is not present in most species of bacteria or higher eukaryotes and has been used clinically for methotrexate toxicity as well as for uncaging of Glu-modified pro-drugs.[5] The ability of CPG2 to hydrolyze urea analogues of Glu provides a unique enzymatic activity to uncage fluorogenic dyes.[5] Based upon the tunable, photostable, and fluorescent properties of 2-dicyanomethylene-3-cyano-2,5-dihydrofuran (CyFur) fluorophores,[6−8] we synthesized a Glu-modified derivative of CyFur (Glu-CyFur) as a potential fluorogenic substrate for CPG2 (Scheme S1). Similar to other acylated derivatives of CyFur that we have previously sythesized,[8] the absorbance and fluorescence emission properties of Glu-CyFur are significantly altered compared to CyFur (Figure 1A and Figure S1). The visible fluorescence emission of Glu-CyFur at 610 nm is reduced approximately 5-fold compared to CyFur (Figure 1A), which is consistent with previously reported quantum yields of CyFur derivatives.[6−8] Importantly, recombinant (r)CPG2 uncaged Glu-CyFur within minutes (Figure 1B), demonstrating that Glu-CyFur is an efficient substrate of CPG2 with a $k_{cat} = 0.25 \pm 0.03$ s^{-1} and $K_M = 1.87 \pm 0.53$ μM (Figure S2). CPG2 is specific for Glu-modified substrates as an aspartic acid derivative, Asp-CyFur, was not uncaged under identical reaction conditions (Figure 1B). The time-dependent uncaging of Glu-CyFur with CPG2 can be readily observed by the naked eye with or without UV-excitation (Figure 1C). These results demonstrate Glu-CyFur is a specific and efficient substrate for CPG2. Notably, the synthesis of Glu-modified fluorophores is concise, high yielding, and potentially compatible with other fluorogenic dyes (Scheme S1).

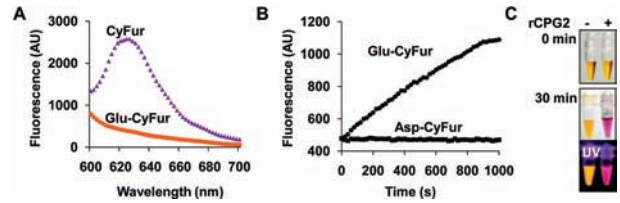


Figure 1. (A) Fluorescence emission spectra of CyFur dyes with 563 nm excitation. (B) Change in fluorescence at 610 nm of CyFur dyes with rCPG2. (C) Visual change in Glu-CyFur fluorescence with rCPG2.

The CPG2:Glu-CyFur reporter system provides a unique opportunity to monitor type III protein secretion from bacterial pathogens. To evaluate protein secretion through the *Salmonella* pathogenicity island-1 (SPI-1) T3SS, CPG2 with a C-terminal HA tag was fused to the C-terminus of an SPI-1 effector SopE2 (Figure 2A). SopE2 is secreted by the T3SS needle complex into growth media or injected into host cells, where its guanine nucleotide exchange factor activity activates Rho-family GTPases to remodel the actin cytoskeleton for *Salmonella* entry. For expression in *S. typhimurium*, SopE2-CPG2-HA was cloned into the pWSK29 plasmid driven by the SopE2 promoter (Figure 2A). Western blot analysis of bacterial cell lysates and concentrated growth media demonstrated that SopE2-CPG2-HA is expressed and secreted by *S. typhimurium* (Figure 2B). Importantly, secreted SopE2-CPG2-HA was enzymatically active as measured by an increase in Glu-CyFur fluorescence (Figure 2C). Glu-CyFur uncaging was not observed for media from bacteria expressing a SopE2-CPG2-HA active site mutant (E175A), even though this protein was expressed and secreted (Figure 2B,C). Confirming that this assay measures secretion through the T3SS, *S. typhimurium* deficient in a structural component of the SPI-1 T3SS needle complex ($\Delta invA$)[10] did not secrete SopE2-CPG2-HA and, consequently, did not exhibit CPG2 activity in the growth media (Figure 2B,C). Immunofluorescence analysis of infected HeLa cells confirms that SopE2-CPG2-HA can be injected into host cells within 30 min (Figure 2D). Glu-CyFur fluorescence uncaging could also be observed for these infected cell lysates (Figure 2E) confirming orthogonality of CPG2 activity to eukaryotic proteomes. These results demonstrate that SopE2-CPG2-HA is stably expressed, is readily

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translocated by the SPI-1 T3SS, and maintains CPG2 activity after secretion. It is important to note that while expression of SopE2-CPG2-HA using a low copy plasmid provides a convenient readout of the SPI-1 T3SS, chromosomal expression of SopE2-CPG2 and normalization for CPG2 fusion will be required to accurately measure endogenous levels of type III protein secretion. Nonetheless, the visible fluorescence detection of Glu-CyFur uncaging provides a very sensitive assay for type III protein secretion in bacterial supernatants and infected cell lysates.

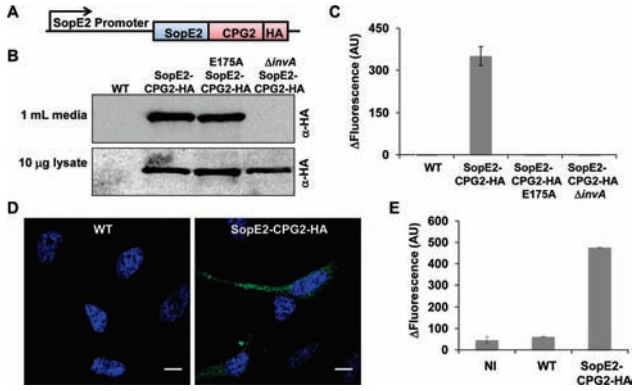

Figure 2. (A) SopE2-CPG2-HA bacterial expression construct. (B) Western blots for SopE2-CPG2-HA expression from concentrated growth media or lysates of indicated strains. (C) Change in CyFur fluorescence over 2 h when 10 μL of growth media from the indicated bacteria strains were added. (D) Visualization of SopE2-CPG2-HA injection into HeLa cells. Cells were infected for 30 min with *S. typhimurium* expressing SopE2-CPG2-HA and stained with anti-HA antibodies (green) and TOPRO-3 reagent (blue). Scale bars indicate 10 μm. (E) Glu-CyFur uncaging in *S. typhimurium* infected HeLa cell lysates.

To explore the sensitivity of the SopE2-CPG2-HA:Glu-CyFur system, we evaluated the activity of reported bacterial T3SS inhibitors with our assay. Salicylidene acylhydrazides (SAHs) such as INP0007 have been shown to block T3SS activity in several Gram-negative bacterial pathogens,[3] including *S. typhimurium*.[11,12] To evaluate T3SS inhibitors with the SopE2-CPG2-HA:Glu-CyFur system, *S. typhimurium* was incubated with INP0007 at increasing concentrations and SopE2-CPG2-HA secretion was measured. Western blot analysis of SopE2-CPG2-HA expression and secretion suggests that INP0007 inhibits bacterial effector secretion at 25 μM without affecting protein expression (Figure 3A). These results are consistent with previous reports of INP0007 activity on proteins secreted by *Salmonella* using coomassie blue staining,[11,12] which we also independently confirmed (Figure S3). Analysis of SopE2-CPG2-HA expression and secretion measuring Glu-CyFur fluorescence suggests 5 μM of INP0007 already significantly inhibits the secretion of SopE2 (Figure 3B) without interfering with protein expression (Figure S4). The inhibitory activity of INP0007 can also be visualized by the naked eye based on the change in Glu-CyFur spectral properties after CPG2 uncaging (Figure 3A).

To investigate whether the SopE2-CPG2-HA:CyFur reporter system could differentially measure the activity of T3SS inhibitors, we evaluated an INP0007 analogue lacking the dibromophenol motif (SAH-1, Figure 3C). Interestingly, SAH-1 showed no inhibitory activity compared to INP0007, which suggests that the dibromophenol motif of INP0007 is crucial for potent inhibition of T3SSs (Figure 3D). INP0007 is ~4 times more potent in blocking SopE2-CPG2-HA secretion than another reported Gram-negative bacteria protein secretion inhibitor, 2-imino-5-arylidene-thiazolidinone (IAT),[13] with IC_{50} values of 5.5 and 22.6 μM respectively (Figure 3D). These experiments highlight the sensitivity of the

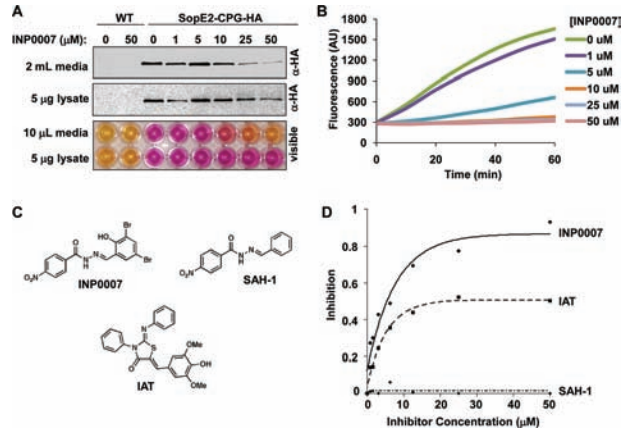

Figure 3. (A and B) *S. typhimurium* strains were grown with varying doses of INP0007 for 4 h. SopE2-CPG2-HA secretion and expression were visualized by western blot and by Glu-CyFur uncaging. (C) T3SS inhibitors. (D) Dose-dependent inhibition of SopE2-CPG2-HA secretion by T3SS inhibitors. For all experiments equal volumes of bacterial growth media were used and comparable bacteria growth was confirmed by measuring optical density at 600 nm.

CPG2:Glu-CyFur reporter system in measuring differential inhibitory activity of small molecules targeted at T3SSs.

The CPG2:Glu-CyFur reporter system provides a robust and sensitive method for monitoring protein expression and secretion. This system is complementary and orthogonal to other enzyme reporter systems such as luciferase and β-lactamase, making it potentially useful for dual-imaging applications in the future.[4,14] Cell-permeable versions of Glu-CyFur may also be useful for live cell and *in vivo* applications. Importantly, the CPG2:Glu-CyFur system provides a simple and rapid means of evaluating cellular factors that influence the secretion of protein toxins and effectors as well as chemical inhibitors for antibiotic development.

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Supporting Information Available: Experimental procedures, supporting figures, and complete refs 7 and 10. This material is available free of charge via the Internet at http://pubs.acs.org.

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